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Forum Review

Redox Reactions of Hemoglobin

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ABSTRACT

Redox reactions of hemoglobin have gained importance because of the general interest of the role of oxidative stress in diseases and the possible role of red blood cells in oxidative stress. Although electron paramagnetic resonance (EPR) is extremely valuable in studying hemoglobin redox reactions it has not been adequately used. We have focused in this review on the important contributions of EPR to our understanding of hemoglobin redox reactions. We have limited our discussion to the redox reactions thought to occur under physiological conditions. This includes autoxidation as well as the reactions of hydrogen peroxide generated by superoxide dismutation. We have also discussed redox reactions associated with nitric oxide produced in the circulation. We have pinpointed the value of using EPR to detect and study the paramagnetic species and free radicals formed during these reactions. We have shown how EPR not only identifies the paramagnetic species formed but can also be used to provide insights into the mechanism involved in the redox reactions. *Antioxid. Redox Signal.* 6, 657–666.

INTRODUCTION

THE NORMAL FUNCTIONAL Fe(II)hemoglobin is a d^6 system and has no electron paramagnetic resonance (EPR) spectrum. Oxyhemoglobin and carbonmonoxyhemoglobin are low-spin complexes and diamagnetic, while high-spin deoxyhemoglobin is EPR-silent because of an even number of unpaired electrons and a short relaxation time. EPR studies on Fe(II)hemoglobin have, however, been performed on complexes with nitric oxide (NO) (10), which is a free radical and, therefore, contributes one additional unpaired electron. Most other EPR studies on divalent hemoglobins have been performed with metal-substituted hemoglobins where the Fe(II) is replaced by Co(II) or Cu(II) (8, 21).

EPR can, however, be used to detect and analyze the Fe(III)hemoglobin (d^5) formed when hemoglobin is oxidized. Because of the short relaxation times of the iron, it is, however, necessary to perform these EPR spectra at low temperatures in the frozen state. While spectra can be obtained at and even above liquid nitrogen temperature (77 K), much better resolution is obtained at lower temperatures approaching liquid helium (4.2 K) (16). The Fe(III) EPR spectrum can distinguish between the spin state of the iron and, particularly in the low-spin state, is very sensitive to the configuration of the ligands around the metal center (29). By detecting only the Fe(III) oxidized hemes, EPR is a sensitive and valuable method for studying hemoglobin redox reactions.

AUTOXIDATION OF HEMOGLOBIN

EPR determination of hemoglobin oxidation

Free ferrous ion is readily oxidized to Fe(III). However, in hemoglobin with the iron chelated by the porphyrin and in a hydrophobic pocket the Fe(II) is much more stable. Nevertheless, the ferrous heme of normal functional hemoglobin continuously undergoes autoxidation (3% of the hemoglobin is oxidized in a 24-h period), producing Fe(III)hemoglobin and a superoxide anion radical:

$$Hb(II)O_2 \rightarrow Hb^+ + O_2^{\bullet -} \qquad (1)$$

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This reaction has been considered to be the source for red blood cell oxidative stress, where the superoxide and secondary reactive oxygen species formed from superoxide are the potential cause of cellular and tissue damage (35). In the red blood cell there are enzymatic systems to reduce the oxidized hemoglobin back to functional Fe(II), necessary for the reversible binding of oxygen. However, there is always a low level of Fe(III)hemoglobin (normally ~1%) present in blood, which represents the steady-state level of Fe(III)hemoglobin that reflects red cell oxidative stress.

Absorption spectroscopy is frequently used to measure the Fe(III)hemoglobin. However, the reliability of these determinations is questionable because of the need to eliminate scattering artifacts of cellular samples, the large preponderance of Fe(II)hemoglobin with overlapping spectra, and the differences in spectral properties of the different Fe(III) states. EPR is, therefore, the ideal method to quantitate Fe(III)hemoglobin (44). Scattering does not affect the EPR spectrum, and packed red blood cells can be directly measured without any contribution from the oxyhemoglobin and deoxyhemoglobin. This method has recently been used to measure oxidation in lipid vesicles where the scattering made it impossible to measure oxidation by visible spectroscopy (2).

The dominant species present in hemoglobin at physiological pH is high-spin Fe(III)hemoglobin with a $g_{\perp} = 6.0$ and a $g_{\parallel} = 2.0$. In determining the oxidation under different conditions it is however necessary to also consider the low-spin signals. Since the low-spin spectrum of an Fe(III) complex consists of three EPR bands and frequently several low-spin complexes with slightly different bands are present at the same time, the low-spin signal heights will be relatively low, but will contribute significantly to the total concentration of Fe(III)hemoglobin. Double integration of the EPR signal using standards with known concentrations of unpaired electrons (44) can be used to quantitate the total Fe(III)hemoglobin. An alternative method involving the binding of a high-spin ligand like fluoride (43) may be able to quantitate the Fe(III)hemoglobin without the need to perform double integration, which can produce artifacts if the baseline is not flat and or other paramagnetic signals are present.

Although the determination of total Fe(III)hemoglobin by EPR is a reliable measure of red blood cell oxidative stress as well as an indirect measure of the reactive oxygen species generated in the blood, it has not been extensively used for this purpose. An indication of the value of this method is the finding of a significant increase in total Fe(III)hemoglobin ($p < 0.0006$) for patients with sepsis (43). It has also been possible to show by EPR that there is a greater level of oxidized hemoglobin in venous blood than in arterial blood (36). This was attributed to increased oxidative processes taking place at low oxygen pressures.

Mechanism of autoxidation

EPR has also been very valuable in studies designed to investigate the mechanism of autoxidation. Hemoglobin oxidation generally involves the reaction of an oxidizing agent with either deoxygenated chains, *e.g.,* ferricyanide (46) or Cu(II) (33), or with oxygenated chains, *e.g.,* phenols (48). Unlike these reactions, autoxidation does not involve the addition of exogenous oxidizing agents and instead utilizes oxygen as the oxidizing agent, with the oxygen being reduced to superoxide. It would have, therefore, been expected that the rate of autoxidation would be proportional to the partial pressure of oxygen. Interestingly (Fig. 1), it has been shown that the rate of autoxidation increases dramatically at intermediate oxygen pressures (1).

This had initially been explained by a bimolecular reaction with oxygen bound as an outer sphere complex to deoxygenated chains (49). While this reaction is necessary to explain the autoxidation of single-chain proteins like myoglobin, it has been suggested that for hemoglobin, increased rates of autoxidation at intermediate oxygen pressures could be explained by the presence of partially oxygenated hemoglobin intermediates with appreciably greater rates of oxidation (34). In this case, the autoxidation involves the oxygen coordinated to the Fe(II)heme instead of a weakly associated oxygen bound to the periphery of the deoxygenated hemes.

In order to establish this mechanism, a novel EPR method was used to trap intermediates in the autoxidation reaction (4). In the temperature range from 200 to 250 K, protein fluctuations take place facilitating reactions that do not require a major rearrangement in protein structure (15, 17). The EPR properties of these intermediates can then be studied at liquid nitrogen or liquid helium temperatures. We had previously used this method to study the formation of reversible bis-histidine complexes in hemoglobin (17). With partially oxygenated hemoglobin (Fig. 2) this method was used to trap the intermediates responsible for the enhanced rates of autoxidation at low oxygen partial pressures (4). By incubating partially oxygenated hemoglobin at 235 K, it was, thus, possible to trap superoxide formed during autoxidation in the hydrophobic heme pocket. At higher temperatures, this superoxide rapidly diffuses out of the heme pocket and dismutates to form hydrogen peroxide and oxygen. However, the demon-

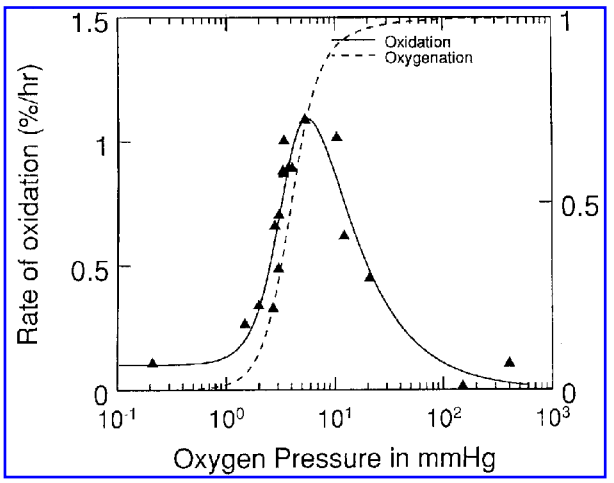

FIG. 1. Rate of oxidation of purified human hemoglobin as a function of oxygen pressure: oxidation (-▲-) and oxygenation (- - -). Conditions included: 0.05 *M* phosphate, pH 7.4, 0.1 m*M* NaCl, 0.1 m*M* EDTA, 25,000 U/ml of catalase, and 75,000 U/ml of superoxide dismutase. Reprinted with permission from Rifkind *et al.* (36).

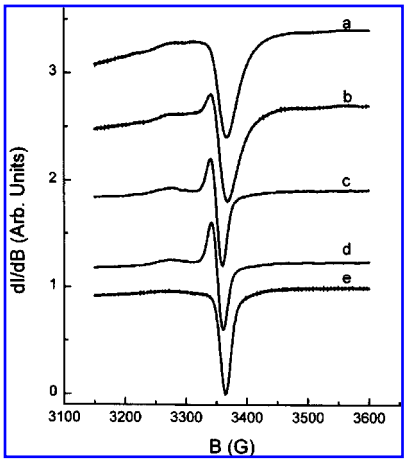

FIG. 2. Evolution and growth of superoxide radical in partially oxygenated hemoglobin (55.1% oxyhemoglobin, 6.7% methemoglobin, and 38.2% deoxyhemoglobin) incubated for different lengths of time at 235 K: 1 min (trace a); 3 min (trace b); 7 min (trace c); and 17 min (trace d). Trace e: Thawed and refrozen sample. The EPR (X-band) spectra (each spectrum is average of six scans) were recorded at 8 K. The $g_{\parallel}$ and $g_{\perp}$ values correspond to 2.0563 and 2.0043, respectively. Reprinted with permission from Balagopalkrishna *et al.* (4).

stration by EPR that superoxide is produced in the frozen state, under conditions where non-bound oxygen cannot diffuse into the heme pocket, indicates that the bound oxygen is converted to superoxide. The relationship of this process to the enhanced rate of autoxidation at intermediate oxygen pressures was established by comparing the oxygen dependence of the rates of autoxidation with the formation of the superoxide radical.

The nucleophilic displacement of bound oxygen

The displacement of superoxide from oxyhemoglobin at intermediate oxygen pressures requires an understanding of the factors that determine the stability of the Fe(II)-oxygen bond in hemoglobin to oxidation. Appreciable electron density is transferred from the Fe(II) to the oxygen, with oxyhemoglobin possessing many of the spectroscopic properties of an Fe(III)-superoxide complex (50). However, there is a delocalization of this electron density, and the superoxide ion does not spontaneously dissociate from oxyhemoglobin. It has, however, been shown that hemoglobin oxidation is facilitated by the nucleophilic displacement of the bound superoxide by nucleophiles like azide and even chloride (49). It has in fact been suggested (47) that in the absence of an added nucleophile, water acts as a nucleophile to displace the oxygen. In the frozen state, where superoxide formation has been observed, an exogenous nucleophile cannot gain access to the heme pocket. How then do we explain the enhanced autoxidation and dissociation of superoxide (1, 4)?

It has been shown that even in the frozen state protein flexibility in the heme pocket results in fluctuations involving the distal histidine (15). In the subzero temperature range from 200 to 250 K, a bond between the heme iron and the distal histidine is stabilized when the fluctuations bring the iron and the N_{ϵ} of the distal histidine close enough to form a bond (Fig. 3). By rapid freezing of methemoglobin, it has in fact been shown that even at room temperature a complex with the distal histidine coordinated to Fe(III) with water still in the heme pocket is present (17). An analogous interaction with oxygen bound to the Fe(II)-heme would result in the nucleophilic displacement of the superoxide ion, resulting in the oxidation of the heme (18).

The nucleophilic displacement of superoxide by the endogenous nucleophile, the distal histidine, thus explains hemoglobin autoxidation. But it is also necessary to explain the oxygen dependence, whereby the autoxidation is enhanced at low oxygen pressures. This has been explained by an increase in distal pocket flexibility in partially liganded hemoglobins (15). An EPR study utilizing valency hybrids (19) has demonstrated that heme pocket dynamics in one subunit is affected by the heme configuration in a second subunit across the $\alpha_1\beta_1$ interface. It was thus shown that replacing oxygen with carbon monoxide in the Fe(II) chain very appreciably alters the distal histidine interactions in the Fe(III) chains (Fig. 4). This was attributed to the change in the orientation of bound oxygen and bound carbon monoxide. In the same way, removing a ligand from some of the hemoglobin subunits in partially oxygenated hemoglobin could facilitate the nucleophilic displacement of superoxide in the oxygenated chains. These subunit interactions explain the Mossbauer results, which indicate increased heme pocket flexibility for partially liganded hemoglobin (15).

Secondary reactions of superoxide in the heme pocket

These studies establish the formation of superoxide in the heme pocket in conjunction with a nucleophilic interaction of the distal histidine with the bound oxygen. The autoxidation

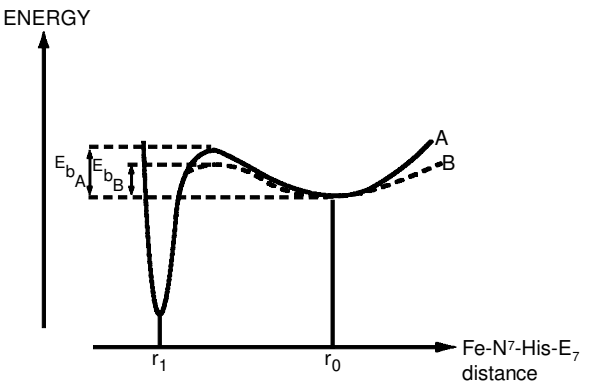

FIG. 3. Potential energy diagram for the formation of a bis-histidine complex involving the distal histidine. The narrow deep well at the left represents the bis-histidine complex with the minimum corresponding to the iron-nitrogen distance of the complex. The broad shallow well represents the iron-nitrogen distance in high-spin deoxyhemoglobin where the histidine is not bound to the iron. Increased flexibility in the heme pocket results in curve B instead of curve A, with a shallower non-bound well and a reduction in the barrier height from E_{bA} to E_{bB}. Reprinted with permission from Levy and Rifkind (15).

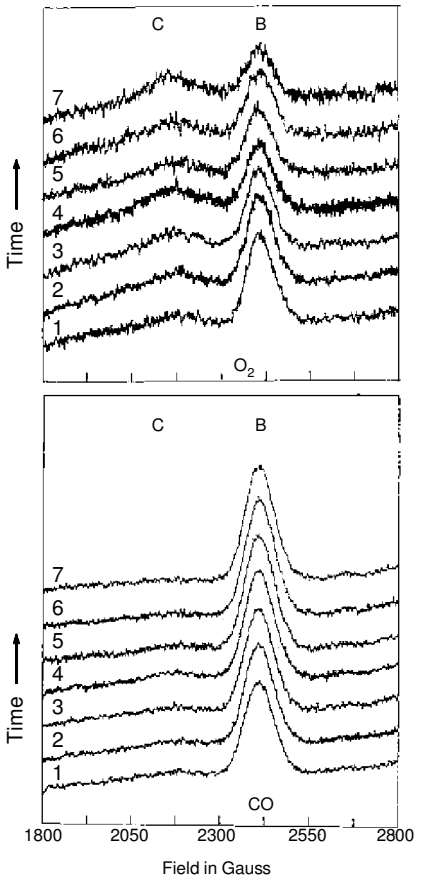

FIG. 4. Effects of incubation at 233 K on the low-spin EPR spectrum (12 K, 0.01 *M* phosphate, pH 6.0) of the (top panel) hybrid ($\alpha^+ \beta^{O2}$) ($\alpha^+ \beta^{O2}$) with both α-chains oxidized and oxygen bound to the chains, and the (bottom panel) hybrid ($\alpha^+ \beta^{CO}$) ($\alpha^+ \beta^{CO}$) with both α-chains oxidized and carbon monoxide bound to the chains. Incubation times were: trace 1, 0 min; trace 2, trace 1 min; trace 3, 2 min; trace 4, 4 min; trace 5, 10 min; trace 6, 20 min; and trace 7, 40 min. Reprinted with permission from Levy *et al.* (19).

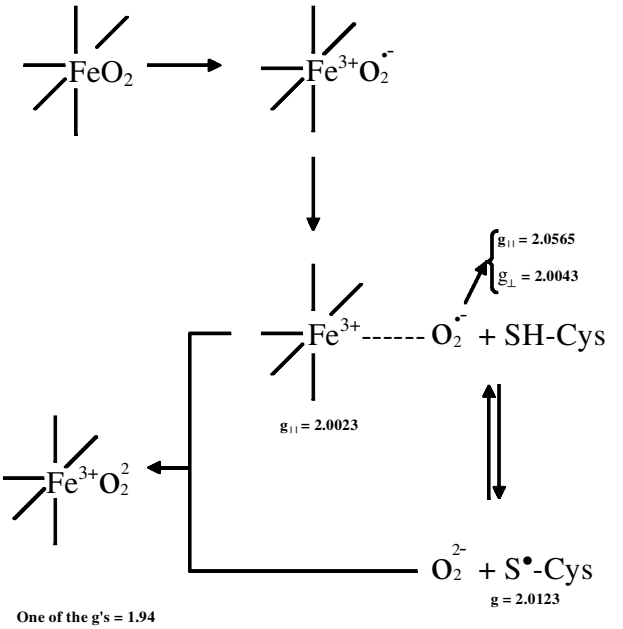

FIG. 5. The overall reaction scheme of hemoglobin autoxidation in the beta chain showing the transfer of an electron from the superoxide to the β-93 cysteine and the formation of a heme peroxy complex.

reaction, however, requires that this superoxide escapes from the heme pocket, producing the relatively stable Fe(III)hemoglobin. Although this reaction is relatively rapid at room temperature, it is nevertheless necessary to consider possible reactions of the superoxide while it is in the heme pocket. It has thus been shown that superoxide can reduce Fe(III)hemoglobin, reforming the Fe(II) heme (40). At the same time the superoxide ion, while in the heme pocket, can react with other groups in the heme pocket. We have thus shown that superoxide can attack the porphyrin, resulting in the eventual formation of heme degradation products (24, 25). This reaction is not very efficient, but does occur to some extent (see below).

We have also shown by EPR studies (5) that in the β-chain with a free cysteine nearby (the β-93 cysteine is on the other side of the heme ~14 Å from the iron) there is an electron transfer reaction between the superoxide and the cysteine, producing a thiyl radical and peroxide, which coordinates with the Fe(III)-heme (Fig. 5). This was demonstrated by a detailed analysis of the changes in the EPR signals during 235 K incubation. After correcting for the high-spin $g = 2$ signal, it was possible to identify the Fe(III)-peroxy complex. At the same time, it was possible to show, by simulation of the free radical signals formed, that for normal hemoglobin, but not for hemoglobin with the cysteine blocked by *N*-ethylmaleimide, it was necessary to include a signal with a g_{iso} = 2.0133 in addition to that of the superoxide. This signal is consistent with that expected for a thiyl radical.

This electron transfer between the superoxide and the cysteine actually stabilizes the intermediate with the superoxide still in the heme pocket and would predict a slower rate for autoxidation in the β-chain than in the α-chain. This prediction explains the finding that in fresh hemolysates, most of the hemoglobin oxidation involves the α-chains. The electron transfer reaction between the superoxide formed during autoxidation and the cysteine thiol group can also have a role in affecting the stability of NO reacted with this thiol group (9).

REACTIONS OF HYDROGEN PEROXIDE WITH HEMOGLOBIN

Hydrogen peroxide is produced as a result of the dismutation of superoxide formed during hemoglobin autoxidation:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (2)$$

Dismutation of superoxide takes place both spontaneously and as a result of the reaction of superoxide dismutase in the red blood cell. Hydrogen peroxide is a two-electron oxidizing agent that can react with both Fe(II)hemoglobin and Fe(III) hemoglobin. The reaction with Fe(II)hemoglobin (deoxyhe-

moglobin or oxyhemoglobin) results in the Fe(IV)ferrylhemoglobin, which has an even number of unpaired electrons and is EPR silent:

$$Hb(II) + H_2O_2 \rightarrow Hb(IV)=O + H_2O \quad (3)$$

The reaction of hydrogen peroxide with Fe(III)hemoglobin produces oxyferrylhemoglobin where the iron is in the +4 oxidation state and an additional electron is transferred from the globin to the heme, resulting in a free radical signal that can be detected by EPR (38):

$$Hb(III) + H_2O_2 \rightarrow {}^{\bullet}Hb(IV)=O + H_2O \quad (4)$$

The globin radical formed by the reaction with Fe(III)

The Fe(II) forms of hemoglobin and myoglobin represent the functional forms of these proteins and are present *in vivo* at much higher concentrations. However, the Fe(III) forms are continuously being formed as a result of autoxidation reactions with other oxidants, as well as the autoreduction of the highly unstable ferrylhemoglobin. It is, therefore, important to also consider the reactions of hydrogen peroxide with Fe(III) heme proteins. There have been extensive EPR studies on the nature of the globin radicals formed during this reaction both in hemoglobin and in myoglobin (41, 45). Spin trapping and low temperature and room temperature EPR of the free radical signals formed have been used to identify the species present. In addition, site-directed mutagenesis and chemical methods have been used to help identify the specific amino acids involved.

It has been shown that both in myoglobin and hemoglobin, tryptophan peroxyl radicals (40) and tyrosine phenoxyl radicals (45) are formed. In human myoglobin, it has further been shown that the tryptophan peroxyl radical initially formed is converted to a thiyl radical (51). The relative amounts of peroxyl radicals and phenoxyl radicals depend on the protein structure. Thus, in horse heart myoglobin the peroxyl radical is the dominant species (41), while in human hemoglobin the phenoxyl radical accounts for 90% of the free radicals formed (45). In human hemoglobin (41) there are two classes of peroxyl radicals. One of them is similar to the peroxyl radical found in myoglobin and is attributed to α-14 tryptophan and β-15 tryptophan, while the other peroxyl radical is thought to involve the β-37 tryptophan. The formation of these peroxyl radicals was shown to depend both on the distance from the heme and on the relative orientation of the heme and the indole ring of tryptophan.

Using site-directed mutagenesis and an analysis of the effect of rotation of the phenoxy ring and the angles found in the X-ray structure, it was concluded that in sperm whale myoglobin the phenoxyl radical is initially formed on tyrosine-103 and migrates to tyrosine-151 (45). Based on an analysis of the room temperature EPR spectrum of hemoglobin A, it was suggested that the phenoxyl radical involves α-Tyr42, β-Tyr35, or β-Tyr130 (45).

The peroxyl radical on both myoglobin and hemoglobin is highly reactive and has been implicated in the oxidation of hydroxybenzoic acid and the catalysis of a number of reactions, including the hydroxylation of anilines, demethylation of arylamines and ethers, oxidation of arylhydrazines and olefins, epoxidation of styrene, and sulfoxidation of chlorpromazine (41). While the tyrosine phenoxyl radical is more stable and has even been detected in whole blood samples (42), it has been implicated in the formation of globin-porphyrin cross-links (31).

The reaction of hydrogen peroxide with Fe(IV) hemoglobins

Hydrogen peroxide not only reacts with Fe(II)hemoglobin and Fe(III)hemoglobin, but also reacts with the Fe(IV)hemoglobins. This reaction with the oxyferrylhemoglobin formed when Fe(III) reacts with hydrogen peroxide is the basis for the catalase activity (14) of Fe(III)hemoglobin. Recycling of Fe(III)hemoglobin occurs when the oxyferryl takes two electrons from hydrogen peroxide, producing oxygen and regenerated Fe(III)hemoglobin. When hydrogen peroxide reacts with the ferrylhemoglobin formed from Fe(II)hemoglobin, two-electron reduction going back to Fe(II)hemoglobin does not occur, and the hydrogen peroxide instead undergoes a one-electron oxidation producing superoxide (26):

$$Hb(IV)=O + H_2O_2 \rightarrow Hb(III) + O_2^{\bullet -} + 2H^+ \quad (5)$$

The formation of this superoxide ion was shown to take place, using EPR (Fig. 6).

Since Fe(III)hemoglobin is always formed during the reaction of hydrogen peroxide as a result of the autoreduction of ferrylhemoglobin, protein radicals are always formed even when starting with Fe(II)hemoglobin. In order to detect the superoxide anion radical, cyanide was added to react with the Fe(III)hemoglobin as it is formed. Cyanomethemoglobin (HbCN) is not seen in the EPR even at temperatures approaching liquid helium, because of the short relaxation time of HbCN. HbCN does not react with hydrogen peroxide, and no oxyferryl protein radicals are detected when cyanide is added to hemoglobin(III) before the addition of hydrogen peroxide. When starting with Fe(II)hemoglobin, the cyanide will react with any Fe(III)-heme produced in the course of the reaction. However, this only partially eliminates the formation of a free radical signal. The residual free radical signal is similar to that detected for superoxide released during autoxidation, which has been attributed to the superoxide formed during the reaction of hydrogen peroxide with ferrylhemoglobin (28).

The formation of superoxide during the reaction of ferrylhemoglobin with hydrogen peroxide, but not during the reaction of oxyferrylhemoglobin with hydrogen peroxide, results in a qualitatively different mode of red blood cell oxidative stress for Fe(II)hemoglobin than that found for Fe(III)hemoglobin. We have already discussed the reactivity of the oxyferryl and its ability to catalyze a variety of oxidative reactions. However, the only process that results in irreversible damage to the heme is the cross-linking of the globin to the porphyrin (31). In fact, increased levels of hydrogen peroxide activate the catalase activity of the oxyferryl, removing the hydrogen peroxide and reducing the reactive oxyferrylhemoglobin back to Fe(III)hemoglobin (14). However, for Fe(II)hemoglobin, excess hydrogen peroxide produces superoxide, and perhaps because of the different heme pocket

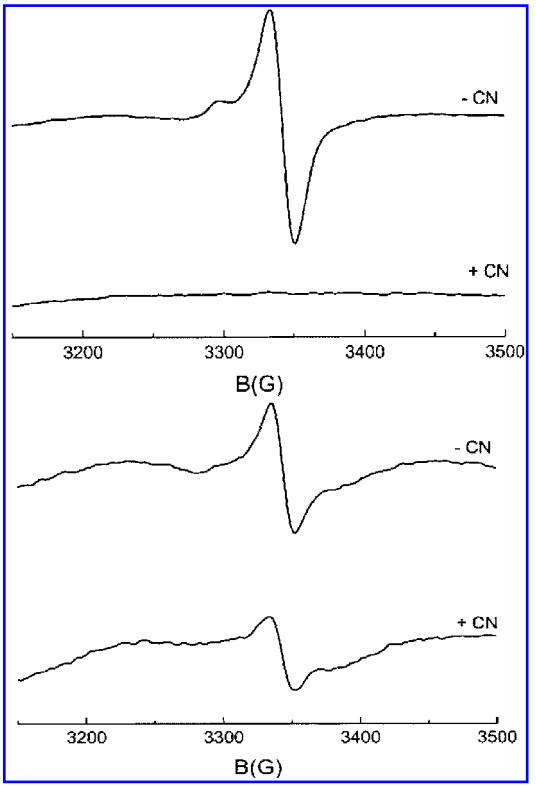

FIG. 6. Effect of cyanide addition on the EPR spectrum of the free radical produced during the reaction of hydrogen peroxide with methemoglobin (top panel) and oxyhemoglobin (bottom panel). The reaction mixture contained methemoglobin (0.4 m*M*) or oxyhemoglobin (0.45 m*M*) in 50 m*M* potassium phosphate buffer (pH 7.4) and 4.5 m*M* H_2O_2 with or without 5 m*M* sodium cyanide (added 15 min prior to initiation of the reaction with H_2O_2). The EPR spectrum was recorded at 10 K on samples quenched into liquid nitrogen 30 s after the reaction with H_2O_2 was initiated. Reprinted with permission from Nagababu and Rifkind (26).

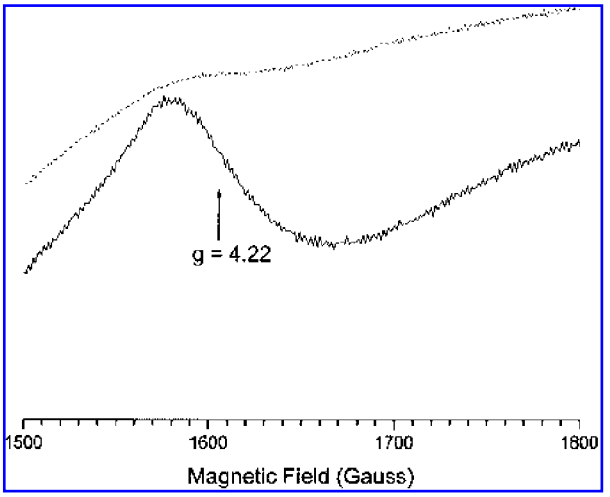

FIG. 7. EPR spectra were recorded at 4 K showing the signal from rhombic heme for hemoglobin A_0 (upper curve) and *O*-raffinose-poly(hemoglobinA_0) (lower curve). Reprinted with permission from Nagababu *et al.* (27).

configuration of ferrylhemoglobin and oxyhemoglobin, this superoxide has a greater tendency to react with the heme than the superoxide formed during autoxidation (24–26).

Formation of high-spin rhombic heme

Using EPR, we have shown that the initial damage to the heme results in a change in the geometry around the iron:

$$Hb^{+}(\text{heme}) + O_2^{\bullet -} \rightarrow \text{High-spin [Fe(III)-rhombic heme]} \quad (6)$$

producing a high-spin rhombic heme with $g = 4.3$ (4) instead of the usual tetragonal high-spin Fe(III) generally found with hemoglobin (Fig. 7). Consistent with the involvement of the superoxide in the production of the rhombic heme complex, no rhombic heme is found when starting with Fe(III)hemoglobin, which does not produce superoxide.

The reaction starting with the formation of rhombic heme eventually results in the release of the iron and the formation of fluorescent degradation products (24). By adding catalase at various time intervals after the addition of hydrogen peroxide, it was possible to show that the production of the fluorescent products requires the reaction of an additional molecule of hydrogen peroxide with the ferrylhemoglobin (26). It was further found that although the addition of catalase decreases the formation of degradation products, it does not completely stop it. This phenomenon was interpreted to mean that it is not the reaction of hydrogen peroxide with ferrylhemoglobin that directly produces degradation products. Instead a reactive molecule produced during this reaction, *i.e.,* superoxide, is responsible for the degradation. Studies on the effect of added catalase on the formation of rhombic heme, in conjunction with determinations of the level of the fluorescent degradation products (authors' unpublished data), indicate that rhombic heme continues to form even after catalase is added, *i.e.,* the reaction involves superoxide and not hydrogen peroxide. Furthermore, the tendency for higher levels of rhombic heme with catalase added suggests that the conversion of rhombic heme into fluorescent degradation products may involve the reaction of a third molecule of hydrogen peroxide (authors' unpublished data):

$$\text{Rhombic-heme} + H_2O_2 \rightarrow \text{Fluorescent products} \quad (7)$$

Both rhombic heme, as detected by EPR, and heme degradation products, detected by both steady-state fluorescence and flow cytometry, have been found in fresh blood samples, indicating that these hemoglobin oxidative reactions occur *in vivo* (authors' unpublished data). While both of these species involve the cascade of the reactions initiated by hydrogen peroxide, they do not necessarily provide the same information. In order to appreciate the relative significance of rhombic heme and fluorescent degradation products, the time course for the formation of rhombic heme and the fluorescent degradation products produced during storage of blood was followed (Fig. 8). We found that the rhombic heme forms first and plateaus off or actually decreases at later times when the fluorescent products begin to be formed [samples indicate

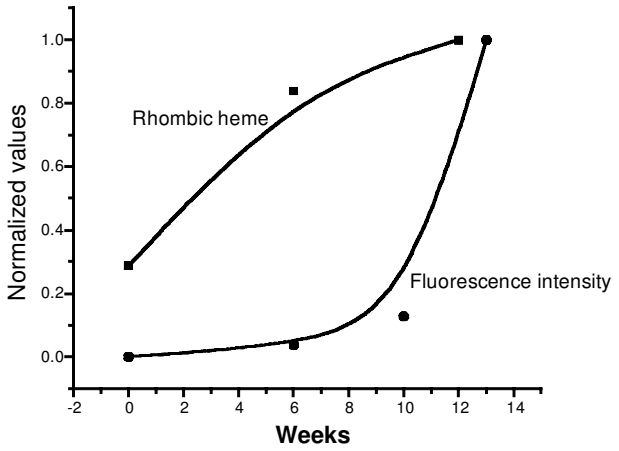

FIG. 8. Comparison of the development of the rhombic heme signal detected by EPR at 4 K(■) and heme degradation products detected by fluorescence spectroscopy at room temperature (●). Between measurements the samples were stored at 4°C. Rhombic heme, which forms faster, is formed during the initial reaction of superoxide with the heme. The fluorescent products are the final products in the oxidative cascade.

that these hemoglobin oxidative reactions occur *in vivo* (authors' unpublished data)]. This time course is analogous to that found for the reaction of hydrogen peroxide with oxyhemoglobin (authors' unpublished data), and is consistent with a reaction pathway where fluorescent products are only formed after the rhombic heme has an opportunity to react with additional hydrogen peroxide. These results suggest that the rhombic heme may provide insight on the initial oxidative processes taking place, while the fluorescent products may be a better way to provide an integral measure of the oxidative stress experienced by the red blood cell.

The presence of rhombic heme and fluorescent degradation products in fresh blood indicates that these secondary redox reactions involving hydrogen peroxide and bypassing the cellular antioxidant enzymes, catalase and glutathione peroxidase, occur even *in vivo*. The primary oxidative reactions involving the autoxidation producing superoxide and the dismutation of superoxide to produce hydrogen peroxide cannot be readily quantitated *in vivo,* because the antioxidant enzymes present maintain a very low steady-state level of these species. However, the secondary oxidative reactions can only occur if autoxidation does take place. By modifying the hemoglobin molecule to alter the stability of ferrylhemoglobin, it was possible to compare hemoglobins being tested as hemoglobin-based blood substitutes (27). Interestingly, diaspirin cross-linked hemoglobin was found to have more than an order of magnitude higher level of rhombic heme than another hemoglobin-based blood substitute, Oxyglobin® (Biopure Corp., Cambridge, MA, U.S.A.), made by intra- and intermolecular cross-linking of bovine hemoglobin with glutaraldehyde. Even though Oxyglobin has a faster rate of autoxidation than the diaspirin-modified human hemoglobin, it had fewer rhombic heme and fluorescent degradation products. Diaspirin-modified hemoglobin has been shown to have a number of toxic effects not found with Oxyglobin and has been withdrawn from clinical trials. This observation suggests that the secondary oxidative reactions may be implicated in some of these toxic side effects.

REACTIONS INVOLVING NO AND ITS METABOLITES

Hb(II)NO

Much of the early EPR studies of hemoglobin involve its reaction with NO (10, 39). NO is a free radical with ligand properties analogous to those of oxygen and carbon monoxide, which bind to Fe(II)hemoglobin. The affinity of NO for Fe(II)hemoglobin is appreciably higher than that for oxygen and even carbon monoxide with the on-rate diffusion controlled and relatively slow off-rate constants (23). Because of the unpaired electron on NO, these complexes have distinctive EPR spectra. The EPR spectra of nitrosylated hemoglobin is, furthermore, sensitive to the heme structure and is different for alpha chains and beta chains (39). The coordination of the NO and the EPR spectra is also dependent on the quaternary conformation with the Fe-proximal histidine bond broken in the alpha chain in the T-state (28). This change in bonding is responsible for a sharpening of the nitrogen hyperfine lines resulting in a distinctive triplet spectrum in the T-state. This spectrum has been used as a measure of the R to T conformational change in hemoglobin. The clear spectral changes associated with conformational change and the slow off-rate for NO have been the basis for the use of NO in the preparation of stable hybrid hemoglobins with ligands bound to some of the hemoglobin subunits (6).

Functional roles of NO

The interest in NO has gained a new dimension with the demonstration that NO is synthesized in the body and acts as a second messenger with many important biological effects. One of the most important biological effects of NO involves the reaction of NO with guanylate cyclase in smooth muscle cells, which produces vasodilatation. The endothelial NO synthase in the vascular endothelial cells provides the NO for this reaction. Some of the NO produced in the endothelial cells also diffuses into the lumen and may play a role in red blood cell function. In fact, a role for hemoglobin in the transport of NO to the vasculature has been proposed (9).

Peroxynitrite

NO is a free radical and is relatively unstable especially in the presence of oxygen. In considering the hemoglobin redox reactions, we have also considered interactions with some of the reactive intermediates formed from NO *in vivo*. NO reacts very rapidly with superoxide (11), producing peroxynitrite ($ONOO^-$). Superoxide is continuously produced in red blood cells as well as other cells. It has, therefore, been suggested that many of the toxic effects attributed to NO are really associated with peroxynitrite. Peroxynitrite can directly react with thiol-containing compounds (37) as well as heme proteins (3, 22, 30). When the peroxynitrite is protonated ($pK_a = 6.6$) it

decomposes to produce nitrogen dioxide and hydroxide radicals (22). The use of EPR to study the interactions with hemoglobin and myoglobin indicates the production of cysteinyl radicals (3) and tyrosinyl radicals (22).

It has been shown that the reaction of peroxynitrite with oxyhemoglobin produces Fe(IV)ferrylhemoglobin (22) with a reaction analogous to that of hydrogen peroxide. It has in fact been suggested (3) that the formation of the tyrosine and cysteine radicals results from an electron transfer reaction between these amino acid side chains and the unstable Fe(IV). However, the ferrylhemoglobin produced from the reaction of hydrogen peroxide does not result in the formation of globin radicals, suggesting that the globin radicals should instead be formed as a result of secondary reactions of the peroxynitrite or its decay products with the globin or the ferrylhemoglobin. In this respect, it is also of interest that excess oxyhemoglobin has been shown to inhibit peroxynitrite-mediated tyrosine nitration of both globin and small substrates (22).

Peroxynitrite also reacts with Fe(III)hemoglobin (30). The evidence for an interaction with the heme is based on the inhibition of nitration of target dipeptides and the observation that the hemoglobin catalyzes peroxynitrite isomerase (30). It is thought that the reaction with Fe(III)hemoglobin goes through the formation of oxyferrylhemoglobin. However, no spectral evidence for the formation of the oxyferryl has been reported, indicating that it must rapidly react with some of the species present regenerating Fe(III)hemoglobin.

Nitrite

NO also reacts with oxygen to produce N_2O_3, which can react with thiol groups and in aqueous solution produces nitrite. It is thought that a large percentage of the 500 n*M* nitrite present in the plasma originates from the reaction of oxygen with NO synthesized in the arteries. Nitrite can diffuse into the red blood cell and react with hemoglobin, oxidizing it (13). However, it has been shown that the reaction with oxyhemoglobin is autocatalytic with a long lag time (12). EPR has been used to delineate the mechanism for this reaction. During the initial phase of the reaction, a free radical signal has been detected. This free radical signal has been attributed to the globin radical associated with oxyferrylhemoglobin. It has been proposed that both iron and nitrite contribute one electron each to oxygen, producing hydrogen peroxide, which reacts with the Fe(III)hemoglobin to produce oxyferrylhemoglobin. The oxyferryl reacts with nitrite to produce methemoglobin and nitrogen dioxide. The autocatalytic nature of the reaction is attributed to a reaction of oxyhemoglobin with nitrogen dioxide regenerating the oxyferryl (20).

As the partial pressure of oxygen is lowered, the nitrite can react with deoxygenated hemoglobin (7) to regenerate NO and Fe(III)hemoglobin. EPR can be used to measure both the Fe(III)hemoglobin and the hemoglobin(II)NO formed during this reaction. As an intermediate in this reaction, NO is bound to the Fe(III)heme oxidized by nitrite (authors' unpublished data). This species can be indirectly quantitated by EPR (32). The high-spin Fe(III) signal is clearly discerned by EPR. However, the binding of NO with an unpaired electron eliminates the EPR signal of the Fe(III). Unlike the Fe(II) complex, which has a very slow off-rate, the NO from the Fe(III)-NO complex can readily be removed by flushing with nitrogen or argon. Therefore, the increase in the Fe(III) signal after the removal of the NO provides a measure of the Fe(III)NO. The possible functional relevance of NO bound to both Fe(II)heme and Fe(III)heme needs to be considered.

CONCLUSION

The use of EPR to study redox reactions in hemoglobin has been reviewed. We have limited our discussion to the redox reactions thought to occur under physiological conditions. This includes autoxidation as well as the reactions of hydrogen peroxide generated by superoxide dismutation. We have also discussed redox reactions associated with NO produced in the circulation. We have pinpointed the value of using EPR to detect and study the paramagnetic species and free radicals formed during these reactions.

ABBREVIATIONS

EPR, electron paramagnetic resonance; HbCN, cyanomethemoglobin; NO, nitric oxide.

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